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*J Immunol* 2005; 175:1177-1183; doi: 10.4049/jimmunol.175.2.1177

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Primed Effect of Lipopolysaccharide on Acetyl-Coenzyme A: Lyso-Platelet-Activating Factor Acetyltransferase Is MyD88 and TRIF Independent

Hideo Shindou,* Satoshi Ishii,** Masahiro Yamamoto,† Kiyoshi Takeda,‡ Shizuo Akira,§ and Takao Shimizu*

LPS has a priming effect on various stimuli. For instance, LPS priming enhances the production of platelet-activating factor (PAF), a proinflammatory lipid mediator that is induced by PAF itself. Among various enzymes responsible for PAF biosynthesis, acetyl-coenzyme A: 1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine acetyltransferase is one of the enzymes activated by PAF receptor stimulation. In this study we investigated the priming effect of LPS on the acetyltransferase activation by PAF in TLR4-knockout (KO) mice, MyD88-KO mice, and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF)-KO mice. This enzyme was biphasically activated by LPS. Although the first peak occurred within 30 min in wild-type (WT), but not TLR4-KO or MyD88-KO, macrophages, the second phase reached a maximum within hours in WT, MyD88-KO, and TRIF-KO, but not in TLR4-KO macrophages. Only in the second phase was the increase in acetyltransferase activity upon PAF receptor activation remarkably enhanced in WT, MyD88-KO, and TRIF-KO cells, but not in TLR4-KO cells. These data demonstrated that LPS exerted a priming effect on PAF receptor-mediated acetyltransferase activation through the TLR4-dependent, but MyD88- and TRIF-independent, pathway. The Journal of Immunology, 2005, 175: 1177–1183.

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Received for publication May 17, 2004. Accepted for publication May 3, 2005.

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1 This work was supported in part by grants-in-aid from the Ministry of Education, Science, Culture, and Sport of Japan (to T.S. and S.I.); Grants-in-Aid for Comprehensive Research on Aging and Health and for Research on Allergic Disease and Immunology from the Ministry of Health, Labor, and Welfare of Japan; and grants from the Yanamochi Foundation for Research on Metabolic Disorders, the Kane Institute for Life and Socio-Medical Science, the Uehara Memorial Foundation (to S.I.), and the Wellcome Medical Research Foundation (to H.S.). H.S. is a research fellow of the Japan Society for the Promotion of Science.

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3 Abbreviations used in this paper: PAF, platelet-activating factor; APMSF, 4-amidino-phenylmethanesulfonyl fluoride; CoA, coenzyme A; cPLA2, cytosolic PLA2; lyso-PAF, 1-O-alkyl-sn-glycero-3-phosphocholine; MEK, MAPK kinase; PLA2, phospholipase A2; mc-PAF, methylcarbamyl PAF; TIR, Toll/IL-1 receptor; TRIF, TIR domain-containing adaptor inducing IFN-β; WT, wild type.

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0022-1767/05/$02.00
LPS PRIMING INVOLVES MyD88- AND TRIF-INDEPENDENT PATHWAY

Worthen et al. (30) previously demonstrated that treatment of human neutrophils with LPS resulted in enhanced production of PAF upon stimulation with fMLP. This LPS-enhanced effect is called priming. Several similar phenomena have been observed. LPS primed the neutrophils such that they responded highly to subsequent fMLP stimulation with enhanced secretion of superoxide anion, O₂⁻ (31), and lysosomal enzymes (32). After stimulation with C5a or fMLP, rabbit neutrophils pretreated with LPS were more injurious to cultured endothelial cells than those without LPS treatment (33). LPS priming in P388D1, macrophage cells enhanced arachidonic acid release and PGE₂ production by stimulants such as PAF (34). Moreover, Heuer et al. (35) showed that pretreatment of mice with LPS enhanced sensitivity to PAF, resulting in increased mortality. However, the mechanisms as well as the molecules involved in the priming effect of LPS remain quite obscure.

Using TLR4-knockout (TLR4-KO) (21), MyD88-KO (36), and TRIF-KO (27) mice, we analyzed the pathway of acetyltransferase activation by LPS. We demonstrated that lyso-PAF acetyltransferase is responsible for enhanced PAF production by the priming effect of LPS, which is MyD88 and TRIF independent.

Materials and Methods

Materials

Methylcarbamyl PAF (mc-PAF), 1-O-hexadecyl-2-N-methylcarbamyl-sn-glycero-3-phosphocholine, and lyso-PAF (1-O-hexadecyl-sn-glycero-3-phosphocholine) were purchased from Cayman Chemical. LPS from Sal-
monella minnesota and 4-amidinophenylmethylene-sulfonfonyl fluoride (APMSF) were purchased from Sigma-Aldrich. [3H]Acetyl-CoA (185 GBq/mmol) was obtained from Amersham Biosciences. SB 203580 and SB 600125 were purchased from Tocris Cookson. U0126 was purchased from Cell Signal-
ing Technology. Recombinant mouse IFN-γ was obtained from Calbiochem. Anti-IFN-γ Ab (clone 7F-D3, a rat IgG1) was purchased from Bio-X-Cell. APMSF, 

Isolation and stimulation of mouse peritoneal macrophages

Three days after i.p. injection of 2 ml of 4% thioglycolate, peritoneal exudate cells were harvested from the peritoneal cavity three times with 5 ml each of ice-cold PBS. The cells were washed twice with 30 ml of ice-cold PBS after centrifugation at 250 × g at 4°C. Total cell numbers were determined with a hemocytometer after staining with Turk solution (Wako). Peroxidase-positive cells were removed by centrifugation at 9000 g, and the cell pellets were washed twice on ice for 30 s each time. Intact cells, cellular debris, and mitochondria were removed by centrifugation at 9000 × g for 10 min at 4°C. The protein concentration of the supernatant was measured by the method of Bradford (37), using protein assay solution (Bio-Rad) and BSA (fraction V, fatty acid-free, Sigma-Aldrich) as a standard.

RT-PCR

TRIF-KO cells were stimulated with LPS for 4 h. After stimulation, total RNA was prepared using the Absolutely mRNA RT-PCR Miniprep kit (Stratagene). Total RNA of macrophages was used as a template to amplify IFN-γ-inducible protein 10 mRNA by IFN through the TRIF-dependent pathway, by OneStep RT-PCR (Qiagen).

Effects of MAPK inhibitors and cycloheximide

Macrophages were pretreated with the indicated concentrations of MAPK inhibitors for 1 h and subsequently stimulated with 100 ng/ml LPS for 30 min. For the cycloheximide experiments, macrophages were pretreated with 10 μg/ml cycloheximide for 1 h and subsequently stimulated with 100 ng/ml LPS for 2 h. The medium was changed and additionally cultured with LPS for 1 h in the absence of cycloheximide, because cycloheximide was toxic to macrophages.

Western blot analysis

Total protein (10.6 μg) in the cell extracts was resolved by SDS-PAGE with 10% polyacrylamide separating gels and transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with Block Ace (Dainippon Pharmaceuticals), probed with anti-

Assay of lyso-PAF acetyltransferase

The assay was modified from the method described by Kume et al. (19). The reaction mixture contained 20 mM Tris-HCl (pH 7.4), 2 mM CaCl₂, 1 mg/ml phosphatidylcholine (Sigma-Aldrich), 100 μM [3H]Acetyl-CoA (1.11 GBq/mmol), 5 mM 2-ME, 20 μM APMSF, a proteinase inhibitor mixture, and 18 μg of proteins with or without 20 μM lyso-PAF to a total volume of 100 μl. The enzyme reactions were performed in a water bath at 37°C. After a 7.5-min incubation, 122 μl of ice-cold methanol was added to terminate the reaction. The products were bound to 6 mg of Bondapak C8 resin, which were washed eight times with 200 μl of 55% methanol in 20 mM Tris-HCl (pH 7.4). The radioactive products were eluted with 250 μg/ml of methanol. After drying up at 50°C for 3 h, the radioactivities were measured with a TopCount scintillation counter (PerkinElmer) in the presence of 200 μl of MesoScint-0 (PerkinElmer). The difference between the radioactivities obtained with and without lyso-PAF corresponded to lyso-PAF acetyltransferase activity.

Priming effect on lyso-PAF acetyltransferase activity

Peritoneal macrophages were pretreated with 100 ng/ml LPS or 100 U/ml IFN-β for the indicated periods and subsequently stimulated with 200 nM mc-PAF for 1 min. The anti-IFN-β Ab (1/500) was added 30 min before LPS treatment.

Statistics

Values were expressed as the mean ± SEM. A value of p < 0.05 was taken to be statistically significant. All statistical calculations were performed with StatView-J, version 5.0 (Abacus Concepts).

Results

Activation of lyso-PAF acetyltransferase in macrophages after LPS stimulation

Peritoneal macrophages from WT, TLR4-KO, and MyD88-KO mice were stimulated with LPS from 0 to 120 min. The activity of acetyltransferase reached a peak at 30 min in WT cells and declined as the incubation continued until 120 min. Neither TLR4-KO nor MyD88-KO cells were responsive to LPS (Fig. 1, A and B). In TRIF-KO cells, however, acetyltransferase was activated 30 min after LPS stimulation at a similar level to WT cells
TRIF-KO cells were practically defective in TRIF-dependent responses to LPS, as judged by the severe reduction of IFN-\(\gamma\)-inducible gene mRNA induction by LPS (data not shown), consistent with previous data (27).

Effects of MAPK inhibitors on the activation of lyso-PAF acetyltransferase

It is known that MAPKs are phosphorylated by LPS stimulation (22, 26, 29). To identify MAPKs involved in the acetyltransferase activation by LPS, we used several specific inhibitors of p38 (SB 203580 and SB 202190), JNK (SP 600125), and MEK1/2 (U0126; Fig. 2). Macrophages were preincubated in the presence or the absence of MAPK inhibitors for 1 h and subsequently stimulated with LPS for 30 min. The LPS-induced acetyltransferase activation was almost abolished in the presence of the p38 MAPK inhibitors SB 203580 and SB 202190, but was unaffected by the inactive analog SB 202474. Consistently, when cell lysates were analyzed by Western blot using an Ab against the phospho- or total form of the p38 MAPK, the amount of phospho-p38 MAPK reached a peak at 30 min after LPS stimulation (Fig. 3). Neither SP 600125 nor U0126 abolished LPS-induced enzyme activation (Fig. 2). LPS-induced phosphorylation of c-Jun and ERK1/2, which are substrates of JNK and MEK1/2, were abolished in the presence of SP600125 and U0126, respectively (data not shown).

Activation of lyso-PAF acetyltransferase in macrophages after PAF receptor stimulation

Lyso-PAF acetyltransferase was previously reported to be activated by PAF stimulation in rat peritoneal neutrophils (9). In peritoneal macrophages from WT, TLR4-KO, and MyD88-KO mice, we examined the elevation of acetyltransferase activity at 0–240 s after PAF receptor stimulation (Fig. 4). In this study we used mc-PAF, a nonhydrolyzable PAF analog, because PAF is degraded by

**FIGURE 1.** LPS-induced activation of lyso-PAF acetyltransferase in peritoneal macrophages. A, WT (■ and □) and TLR4-KO (● and ○) cells were stimulated with (■ and ●) or without (□ and ○) 100 ng/ml LPS for 0, 7.5, 15, 30, 60, and 120 min. Enzyme activity was measured as described in Materials and Methods. One hundred percent acetyltransferase activities represent 97 (WT, vehicle), 73 (WT, LPS), 147 (TLR4-KO, vehicle), and 127 (TLR4-KO, LPS) pmol/min/mg protein. B, WT (■ and □) and MyD88-KO (● and ○) cells were stimulated and assayed by the same procedure as in A. One hundred percent acetyltransferase activities represent 165 (WT, vehicle), 129 (WT, LPS), 127 (MyD88-KO, vehicle), and 139 (MyD88-KO, LPS) pmol/min/mg protein. C, WT and TRIF-KO cells were stimulated with (■) or without (□) 100 ng/ml LPS for 30 min. One hundred percent acetyltransferase activities represent 141 (WT, vehicle) and 112 (MyD88-KO, vehicle) pmol/min/mg protein. Lyso-PAF acetyltransferase was activated through the TLR4/MyD88-dependent pathway, but not through the TRIF-dependent pathway. The results are expressed as the mean ± SEM of a single experiment performed in triplicate. All experiments were performed independently three times with similar results.

**FIGURE 2.** Inhibition of LPS-induced lyso-PAF acetyltransferase activity in peritoneal macrophages. Cells were incubated with 20 \(\mu\)M SB 203580 (\(n = 5\) experiments, performed in triplicate), 20 \(\mu\)M SB 202190 (\(n = 5\)), 20 \(\mu\)M SB 202474 (\(n = 5\)), 10 \(\mu\)M SP 600125 (\(n = 4\)), or 20 \(\mu\)M U0126 (\(n = 4\)) for 1 h and then stimulated with 100 ng/ml LPS for 30 min. One hundred percent represents the specific activity of the enzyme ranging between 107 and 163 pmol/min/mg protein. LPS-induced acetyltransferase activation was almost abolished in the presence of the p38 MAPK inhibitors SB 203580 and SB 202190. Statistical analyses were performed using ANOVA with Scheffe’s test (*, \(p < 0.05\)).

**FIGURE 3.** Activation of p38 MAPK in peritoneal macrophages after PAF receptor stimulation

Lyso-PAF acetyltransferase was previously reported to be activated by PAF stimulation in rat peritoneal neutrophils (9). In peritoneal macrophages from WT, TLR4-KO, and MyD88-KO mice, we examined the elevation of acetyltransferase activity at 0–240 s after PAF receptor stimulation (Fig. 4). In this study we used mc-PAF, a nonhydrolyzable PAF analog, because PAF is degraded by

**FIGURE 4.** Activation of p38 MAPK in peritoneal macrophages after PAF receptor stimulation

Lyso-PAF acetyltransferase was previously reported to be activated by PAF stimulation in rat peritoneal neutrophils (9). In peritoneal macrophages from WT, TLR4-KO, and MyD88-KO mice, we examined the elevation of acetyltransferase activity at 0–240 s after PAF receptor stimulation (Fig. 4). In this study we used mc-PAF, a nonhydrolyzable PAF analog, because PAF is degraded by
PAF acetylhydrolases in serum (38, 39). In all genotypes, the enzyme was rapidly activated, with a maximum at 30 s after mc-PAF stimulation. The mc-PAF-induced enzyme activation was not suppressed by any of the inhibitors of p38, JNK, or MEK1/2 (data not shown).

**Priming effect of LPS on mc-PAF-induced acetyltransferase activity**

WT macrophages were treated with LPS for 1, 4, 8, and 16 h. At each time point, the cells were stimulated with mc-PAF for 1 min (Fig. 5A). This procedure reveals the priming effect of LPS on PAF receptor-mediated acetyltransferase activation. We found that such a lengthy (8 and 16 h) treatment of cells with LPS alone elicited a second-phase increase in the enzyme activity. Even though the response to mc-PAF alone remained constant during the assay, LPS markedly potentiated the enzyme activation by mc-PAF at 16 h. Acetyltransferase activities were increased 4.1/0.7-, 2.8/0.3-, and 9.7/1.5-fold (mean ± SEM; n = 6) in LPS alone, mc-PAF alone, and both stimulations, respectively, compared with nonstimulated cells. Although the enzyme was biphasically activated at 30 min and 16 h after LPS treatment (Fig. 1 and 5, A and B), the priming effect of LPS on PAF was only observed at 16 h and was absent at 30 min (Fig. 5B). The priming effect of LPS as well as the LPS-induced acetyltransferase activation were almost abolished by cycloheximide (Fig. 5C).

**Priming effect of LPS in the MyD88- and TRIF-independent pathway**

Unlike WT macrophages, LPS treatment did not enhance PAF receptor-mediated acetyltransferase activation in TLR4-KO macrophages (Fig. 6A). However, LPS-treated MyD88-KO cells and TRIF-KO cells displayed a marked increase in the enzyme activity by mc-PAF, at a similar level to WT cells (Fig. 6, B and C). A low dose of LPS (1 ng/ml) also displayed the priming effect in both MyD88-KO and TRIF-KO cells at a similar level to WT cells (data not shown).
Discussion

In this report we indicated that lyso-PAF acetyltransferase was activated 1) by a second-order time course after PAF receptor stimulation, 2) by a minute-order time course after LPS stimulation in the MyD88- and p38 MAPK-dependent pathway, and 3) by an hour-order time course after LPS-stimulation in the MyD88- and TRIF-independent pathway (Figs. 6, B and C, and 7A). Furthermore, this study demonstrates for the first time that the LPS-priming effect on acetyltransferase activation occurs through the TLR4-dependent, but MyD88- and TRIF-independent, pathway (Fig. 7).

Although the activity of lyso-PAF acetyltransferase reached a peak at 30 min with LPS treatment in WT cells, neither TLR4-KO nor MyD88-KO cells displayed the enzyme activation by LPS (Fig. 1, A and B). These results indicate that the TLR4/MyD88-dependent pathway is responsible for LPS-induced acetyltransferase activation. TLR4 binds MyD88 via TIR domain-containing adaptor protein (28). MyD88 possesses the death domain, which mediates the association with IL-1R-associated kinase, which is then phosphorylated. The phosphorylated IL-1R-associated kinase associates with TNFR-activated factor 6 and activates the MAPKs. This process leads to the activation of p38 MAPK (22–24). We showed in this study that LPS-induced acetyltransferase activation was almost abolished in the presence of p38 MAPK inhibitors (Fig. 2). In addition, phosphorylation of p38 MAPK peaked at 30 min after LPS stimulation (Fig. 3) in parallel with acetyltransferase activity (Fig. 1). These results demonstrate that lyso-PAF acetyltransferase of macrophages is activated by LPS stimulation in the p38 MAPK-dependent pathway. In MyD88-KO macrophages, although production of cytokines, IL-1β, TNF-α, and IL-6 in response to LPS was completely impaired, activation of p38 was delayed (from 10 to 20 min) but not attenuated, compared with WT (26, 29). However, our study showed that lyso-PAF acetyltransferase of MyD88-KO macrophages was not activated, at least within 120 min after LPS stimulation (Fig. 1B). In human macrophages, expressions of p38α and -δ mRNA were high, but those of β and γ mRNA were low (40). Additionally, SB 203580 and SB 202190 inhibited the enzymatic activity of p38α and -β isoforms, but did

FIGURE 6. Priming effect of LPS on mc-PAF-induced acetyltransferase activity in TLR4-KO, MyD88-KO, and TRIF-KO macrophages. TLR4-KO (A), MyD88-KO (B), and TRIF-KO (C) macrophages were incubated with 100 ng/ml LPS for 16 h, followed by stimulation with 200 nM mc-PAF for 1 min. WT, MyD88-KO, and TRIF-KO cells, but not TLR4-KO cells, exhibited a priming effect. The results are expressed as the mean ± SEM of a single experiment, performed in triplicate. Statistical analyses were performed using ANOVA with Fisher’s protected least significant difference test (*, p < 0.05). Experiments were performed independently three times with similar results.

not for $\gamma$ or $\delta$ (41, 42). Thus, our data suggest that lyso-PAF acetyltransferase is activated mainly by the p38 isoform, p38$\alpha$ is unlikely to be activated by the MyD88-independent pathway.

By mc-PAF stimulation, lyso-PAF acetyltransferase was rapidly activated in WT, TLR4-KO, and MyD88-KO macrophages, with a maximum at 30 s (Fig. 4). These results suggest that the enzyme is normally expressed and activated by PAF in TLR4- and MyD88-KO mice. The peak of acetyltransferase activation by mc-PAF stimulation occurred much earlier than that by LPS stimulation. Furthermore, mc-PAF-induced acetyltransferase activation was not suppressed by any of the inhibitors of p38, JNK, and MEK1/2. PAF appears to activate lyso-PAF acetyltransferase in a MAPK-independent, and possibly Ca$^{2+}$-sensitive manner.

Furthermore, mc-PAF-induced acetyltransferase activation was not suppressed by any of the inhibitors of p38, JNK, and MEK1/2. PAF appears to activate lyso-PAF acetyltransferase in a MAPK-independent, and possibly Ca$^{2+}$-dependent manner (43, 44). However, cellular signaling to acetyltransferase activation by PAF remains to be clarified.

To investigate the priming effect of LPS, WT macrophages were treated with LPS for >8 h and were subsequently stimulated with mc-PAF (Fig. 5A). We found that such a long treatment with LPS alone elicited the second phase of acetyltransferase activation. Furthermore, LPS markedly potentiated the acetyltransferase activation by mc-PAF at 8 and 16 h. This kind of biological response has been called LPS priming (30–35). Although lyso-PAF acetyltransferase was biphatically activated 30 min and 16 h after LPS treatment (Fig. 1, A, B, and C), the priming effect of LPS on PAF was only observed at the second phase (Fig. 5B). It is noteworthy that both MyD88-KO cells and TRIF-KO cells exhibited such a priming effect; LPS-treated MyD88-KO cells and TRIF-KO cells displayed marked increases in the acetyltransferase activity produced by mc-PAF at levels comparable to WT cells (Fig. 6, B and C). These results showed that MyD88 and TRIF were dispensable for LPS priming of mc-PAF-induced acetyltransferase activation. MyD88 is a critical adaptor protein for TLR4 signaling (22–25). These results showed that MyD88 and TRIF were dispensable for LPS priming of mc-PAF-induced acetyltransferase activation. MyD88 is a critical adaptor protein for TLR4 signaling (22–25). Additionally, studies are required for elucidation of the pathways and molecules downstream of TLR4.

Mariano et al. (46) showed that blood and urinary concentrations of PAF were significantly higher in septic patients than in control subjects. In humans administered LPS treatment, with the PAF receptor antagonist Ro 24–4736 resulted in fewer symptoms, such as rigor and myalgia, than those with a placebo (47). These results suggest the clinical significance of PAF in sepsis. We recently demonstrated the importance of cytosolic PLA$_2\alpha$ (cPLA$_2\alpha$) in a mouse model of adult respiratory distress syndrome caused by sepsis, using both cPLA$_2\alpha$-deficient mice (48) and a cPLA$_2\alpha$ inhibitor (49). Because cPLA$_2\alpha$ is also a crucial enzyme for PAF synthesis via the remodeling pathway (12, 50), it is possible that PAF is involved in the pathology of sepsis-induced adult respiratory distress syndrome. Therefore, many symptoms of sepsis may be ameliorated by restricting the MyD88- and TRIF-independent pathway that leads to PAF production by LPS priming.

Acknowledgments
We are grateful to Drs. T. Nagase, T. Yokomizo, N. Uozumi, and H. Nishina (University of Tokyo) for valuable suggestions, and to K. Naguchi, C. Ohkawara, T. Sato, Y. Takahashi, Y. Yamada, Y. Kitaie, and F. Hamano (University of Tokyo) for technical assistance. We also thank John H. Jennings for critical reading of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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